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A Method to Diagnose Retinoblastoma in Children

[SPOSOB DIAGNOSTIKI RETINOBLASTOMY U DETEY]

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(54) A METHOD TO DIAGNOSE RETINOBLASTOMA IN CHILDREN

(57) **Abstract.** The invention concerns oncoophtalmology and it may be applied for differential diagnostics of retinoblastoma. The purpose of this invention is simplify the method, reduce it complexity and improve its accuracy based on differential diagnostics of retinoblastoma at

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[1]Numbers in the margins indicate pagination in the foreign text.

nonneoplastic eye pathologies. The above objective is attained based on incorporation of immunology delay-type hypersensitivity reactions using aqueous saline retinoblastoma cell extracts into examinations of patients. The new features of this method include testing *in vitro,* and also determination of migration capacity of peripheral blood leucocytes using a number of water-soluble antigen preparations of retinoblastoma tissue biopsy material; the preparations contained polypeptides (50 - 100 kD in molecular mass) at 100 - 200 μg/ml concentrations over time interval from 16 to 22 hrs from the beginning of the migration reaction. This method allows to improve the accuracy of pre-surgical diagnostics both for the early and late stages of retinoblastoma development in primary patients, detect growth of tumor on the retina of a "better eye" after removal of the second, "bad" eye due to retinoblastoma, and also reduce traumatism and possibility of complications.

The invention concerns the field of medicine, specifically, oncoophtalmology, and may be applied for differential diagnostics of retinoblastoma at nonneoplastic eye damages.

The purpose of this invention is simplify the method, reduce it complexity and improve its accuracy based on differential diagnostics of retinoblastoma at nonneoplastic eye pathologies.

The delay-type hypersensitivity reaction is realized *in vitro* to determine the migration capacity of peripheral blood leucocytes using water-soluble antigens (50 - 100 kD in molecular mass) extracted from biopsy material of retinoblastoma tissues.

The method is realized as follows:

Migration capacities of the peripheral blood leucocytes in the presence of retinoblastoma tissue antigen preparations are determined with the leukocyte migration inhibition test.

Leukocyte migration indices (MI) are calculated in percent. All the primary untreated patients are diagnosed as having retinoblastoma at MI values lower than 95 %. In cases where the MI values are equal to or higher than 95 % the patients

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(primary untreated patients) are diagnosed as having nontumor pathologies.

The antigen preparation for our diagnostics is obtained as follows:

Retinoblastoma tissues obtained after enucleation of eye and kept at 20°C are unfrozen, weighed and homogenized in ice environment, in a physiologic saline of sodium chloride at pH 4.5 - 5.0 and 1:9 tissue-to-solution weight ratio. The product of homogenization is filtered through a caprone mesh. The filtrate is cooled down to 2 - 4 °C and, after determination of its volume, at pH 4.5 - 5.0 potassium chloride is added at continuous mixing up to 3 M its final concentration in the filtrate. The extraction proceeds for 16 hrs under continuous mixing with a magnetic stirrer. Then

the material is centrifuged at 20,000 rev/min for 30 minutes at 4 °C. Potassium chloride and invaluable constituents without diagnostic activity (lower than 50,000 D in molecular mass) are eliminated from the resulting supernatant with an extraction column filled with Sephadex G-75. The specimen (4 to 5 ml) is placed on a 4.0 cm x 15 cm column, and elution is made with distilled water. The void volume fractions (more than 50,000 D in molecular mass) are combined and lyophilized. The preparation is used for the leukocyte migration tests in the agar blocks at 100 to 200 μg/ml concentrations by proteins.

The leukocyte migration inhibition test is realized as follows: Firstly, should be prepared a 3 % "Difco" agar in 0.05 M *tris*-HCl buffer solution containing 0.9 % of NaCl at pH = 7.3. A volume of 3 % agar at 50-55° C is mixed with two volumes of the 199 medium, which contains 1.5 % of human serum, Group IV, and then put into plastic cups up to 4 mm thickness of agar layer. Once the agar congealed, the cups are placed into a wet-environment desiccator in atmosphere of 5 % concentration of CO_2. Then, the desiccator is allowed to stay for no less than 2 hrs at 4 °C. Holes (three holes

for reference samples with no antigen and those for antigen-containing samples arranged in agar blocks 3 each) are made in the agar blocks with a punch, 3 mm in diameter. Up to the test reaction the agar blocks are kept in a wet chamber at 4 °C in the atmosphere of 5 % CO_2. Venous blood taken from each patient (4 to 5 ml) is placed into tubes with 0.5 ml of 2.7 % solution of Trilon B (a chelating agent). Subsequent to erythrocyte sedimentation the erythrocyte-enriched blood plasma is drawn off and centrifuged for 5 minutes at 1,200 rev/min. The leykocyte sediment is washed out two times with the Hanks' (balanced salt) solution (not containing any calcium or magnesium ions) under the same conditions. Once the leukocyte concentration has been brought up to 2.5 x 10^7, the suspension in place into holes, 10 µl per a hole. Next, Hanks' solution is placed into (10 µl each) reference holes; the diagnostic retinoblastoma tissue antigen preparation, which contains 100 to 200 µg of proteins per 1 ml of the Hanks' solution, is placed into test holes (10 µl each). The cups with leukocytes in agar blocks are placed into a wet desiccator in the atmosphere of 5 % CO_2. The dessicator is enclosed in constant-temperature cabinet for 17 hrs at 37 °C. Right after incubation the cups with agar

are filled with 10 % solution of formaldehyde for 1 hr for the purpose of cell fixation. Once Formalin drained, the agar is dried a little up to disappearance of moisture in holes, and then removed. Now we have to assess the results of the reaction. Average widths of the leukocyte migration zones for reference holes (l_k) and antigen holes (l_a) are determined as distances from the boundaries of these holes to those of the migration zone. The leukocyte migration index is calculated by a formula:

$$MI = \frac{I_a}{I_k} \times 100\ \%.$$

All the resulting data have been statistically processed, and average MI values calculated for the groups of examined sick children as follows: Group I - primary retinoblastoma (Stages I-IV) patients (8 children). MI = 79 ± 12 %; the diagnosis has been confirmed by histology examinations; Group 2 - patients having recurrent retinoblastoma on their second eyes after enucleation of their first "bad" eyes, Stages I and II of the process (12 children). MI = 89 ± 2 %; the diagnosis has been confirmed by histology examinations

based on the removed "bad" eyes; Group 3 - pre-surgery primary patients reliably clinically diagnosed of retinoblastoma with the instrumental methods and ophtalmoscopy, Stages I and II of the disease (16 children). MI = 89 ± 2 %; enucleation was completed diagnosis has been confirmed by histology examinations for six children. Group 4 - reference group of children having nonneoplastic eye pathologies; the patients were considered suspicious for retinoblastoma; however their retinoblastoma diagnoses were not confirmed based on positive dynamics or by histology examinations after enucleations of damaged eyes (due to blunders of instrumental diagnostics); average value of MI = 108 ± 7.5 %; composition of the reference group based final diagnosis: chorioretinitis (2 children); retinitis (2 children); Coats' retinitis (2 children); cataract and vitreous body fibrosis, congenital eye grounds pathology, consequences of uveitus (8 children; two of them with wrong diagnoses: retinoblastoma was not confirmed by histology examinations after eye removal); Group 5 - sick children having uveitis in its acute form (7 patients). MI = 100 ± 2.5 %.

Based on the Wilcoxon-Mann-Whitney test, the MI values for Groups 1 to 3 differ from those for the Groups 4 and 5 with a high degree of certainty ($P < 0.001$). For one patient of 36 children in Groups 1 to 3 (2.8 %) the MI was equal to 96 %, i.e. it was just at the upper limit of the diagnostically significant parameter. For one patient of 23 children of the reference group (4 %) the MI was equal to 95 %, i.e. was at the lower limit for the reference group. Enucleations based on computerized tomography data where no retinoblastoma found after histology verifications were completed in two of 23 children of the reference group,(MI = 100 %; MI = 105 %; 9 % of patients).

Case Study 1. A female patient XX, year of birth - 1980 (examined in 1982). Diagnosis - bilateral retinoblastoma, Stages I to II. [OD … three words illegible - Translator's Note] … bilateral retinoblastoma, removed from both eyes. The child's mother has congenital cataract. Brightness of the child's left eye (OS) pupil was detected at her age of two. At admission to the clinic: the front section of right eye (OD) not changed; in the lower area of the eye ground

from 5 to 7 hrs: neoplasm tissue, white-to-gray colored, showing slight prominence into the vitreous body; the peripheral boundary not ophtalmoscopic. The left eye: the eye chamber is filled up completely with new-generated tissue. Based on ultrasound examination: possible bilateral retinoblastoma. On August 07, 1982: completed examination with the proposed method of leukocyte migration testing using the retinoblastoma antigen preparation. Venous blood (5 ml) drawn from the patient was placed into a tube containing 0.5 ml of Trilon B (2.7 % solution) at pH = 7.3. This blood was allowed to settle for 40 minutes; blood plasma drawn after erythrocyte sedimentation was centrifuged at 1,200 rev/min for 5 minutes; the cell sediment was re-suspended in Hanks' (balanced salt) solution (not containing calcium and magnesium) at pH = 7.3, and then re-settled two times under the same centrifuge conditions. The cell sediment was re-suspended in 2 ml of Hanks' solution; the concentration and total number of extracted leukocytes was counted with a Gorjaev's count chamber (7.5×10^6). The suspension was centrifuged under the same conditions, and cell sediment re-suspended in 0.3 ml of Hanks' solution to reach the 2.5×10^7 concentration. All holes made in the prearranged agar blocks (1 % of Difco agar and 1 % serum of

human blood, Group IV in 19^9 medium) were filled with the suspension, 10 ml each, i.e. 2.5 x 10^5 cells per a hole. Next, Hanks' solution was placed into three reference holes (10 µl per a hole); the antigen preparation obtained from retinoblastoma tissue with the above method, which contained 100 µg/ml of proteins (in the Hanks' solution), was placed into three other holes of the came cup (10 µl per a hole). The incubation was completed at 37 °C for 17 hrs in a wet chamber in the atmosphere of 5 % CO_2. Once the reaction stopped, each cup, 3.5 cm in diameter, was filled in 2 ml of formaldehyde (10 % solution) for the purpose of cell fixation. Next, formaldehyde was drained, and agar dried a little, and then removed. Average width values widths for the leukocyte migration zones were determined for the reference and antigen holes with a binocular microscope. The calculated leukocyte migration index (MI = 82 %) points to inhibition of the leukocyte migration activity, thus reflecting a specific response of T-lymphocytes to the retinoblastoma antigens. Diagnosis: Retinoblastoma. This diagnosis has been confirmed later by histological examinations after enucleation of the "bad" eye. Examinations based on this procedure were completed with a

"blind" method. The patient has been classified into Group I.

Case Study 2. A male patient XY, year of birth - 1976. Diagnosis at admission to the clinic - congenital pathology of both eyes, microphtalmia, corneal caligo, changes in the eye grounds, nystagmus; suspicions for retinoblastoma: cornea with large-scale opacity zones, a few synechias, atrophic iris, clouding of the crystalline lens; vitreous body not ophthalmoscopic, no reflex on the fundus of eye. Ultrasound examination data: isolated "migrating clouds" in the vitreous body; no detachment of retina. The patient was examined with the proposed method on May 20, 1982. All the procedures were completed in similar way as those in Case Study 1. The calculated MI was equal to 114 %. The migration activity of leukocytes in response to the antigen preparation suited to the standard. No negative clinical diagnostics was found later for this case. Final clinical diagnosis: consequences of antenatenal uveitus. The patient has been classified into the reference group as that having nonneoplastic pathology of the retina (Group IV).

Case Study 3. A male patient YY. Diagnosis based on computerized tomography and echography examinations: unilateral retinoblastoma. November 11, 1986: Examinations were completed following the proposed procedure with a "blind" method. All the procedures were the same as those for Case Study 1. The determined leukocytes IM = 100 %. Enucleation of the eye with suspicions for retinoblastoma

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was completed based on the data of clinical instrumental diagnostics. The histological examinations have not confirmed the "Retinoblastoma" diagnosis: however, their findings matched the data completed in compliance with our proposed method, which ruled out the possibility for retinoblastoma to exist. Final diagnosis: consequences of the uveitus. The patient has been classified into the reference group (Group IV).

Case Study 4. A female patient YZ. The patient was diagnosed with "Retinoblastoma" based on echography and computerized tomography examinations. The patient was examined with the

proposed method on February 24, 1988. All the procedures were completed in similar way as that in Case Study 1 with a "blind" method. The calculated MI was equal to 115 %. The patient's eye was removed based on results of instrumental diagnostics. The histological examinations have not confirmed the "Retinoblastoma" diagnosis: however, the findings matched the data completed in compliance with our proposed method: "No retinoblastoma". Final diagnosis: consequences of the uveitus. The patient has been classified into the reference group (Group IV).

Final diagnosis: consequences of the uveitus. The patient has been classified into the reference group (Group IV).

Case Study 5. A female patient ZZ, year of birth - 1986. Diagnosis: bilateral retinoblastoma, Stages I to II. At admission to the clinic: OD (right eye) - degree of prominence - 4.5 mm; damaged area - up to 50 %; OS (left eye) - degree of prominence - 3.0 mm; damaged area - 50 %; Retinoblastoma could not be ruled out based on ultrasound examinations. The patient was examined with the proposed method based on the leukocyte migration test using the retinoblastoma tissue antigen preparation on April 20, 1988. All the procedures were completed in similar way as that in

Case Study 1 except for the fact that the leukocyte concentration was adjusted to 5.0×10^7; leukocytes were added at a dose of 5.0×10^5 per a hole, and concentration of the retinoblastoma antigen preparation was equal to 200 μg per a ml. MI was equal to 90 %. The second immunology examination was completed on October 12, 1988. The MI was equal to 82.5 %. Examinations completed just before the operation: the "bad" eye: degree of prominence - 9.0 mm; damaged area - 80 to 90 %. Immunology testing data: Mi = 85 - 89 %. The right eye of the ZZ patient was removed. Histological examination has confirmed the "Retinoblastoma" diagnosis.

The application of the above method to practice will improve the accuracy of diagnostics for the early (Stages I - II) and late (Stages III - IV) of retinoblastoma development in the primary untreated patients, and also to help to detect tumor growth on the second eye after removal of the "bad" one due to retinoblastoma. Our method is characteristic of high specificity just because it is based on the specific response of the T-lymphocytes to retinoblastoma antigens; specificity of the reaction has been demonstrated in the known procedure at the application of skin tests. Our method

does not require any cost-expensive systems/facilities; further, it is simple and highly reproducible.

To assess the specificity of the antigen preparations we have examined children of the same age division having lymphatic proliferation diseases, specifically malignant disorders: lymphogranulomatosis (Hodskin's disease), lymphatic leukemia, myeloleukemia, malignant lymphoma, and also those having benign lymphatic proliferations (lymph node adenitis, tonsillitis) as well as visibly healthy children. The individual MI values in the reference groups were within the allowable limits or higher than 100 % (within the 2 - 4 % MI error). The MI values for the reference group, both average and individual, were reliably different from those for the retinoblastoma children ($P < 0.001$ according to the Wilcoxon-Mann-Whitney test). Our data have validated the high specificity of retinoblastoma antigen preparations, which are used for diagnostics with the our proposed method.

The Claim

The method of retinoblastoma diagnostics in children through clinical examinations and completion of a certain immunology reaction for the antigen preparations of retinoblastoma **distinctive by its features that,** for the purpose to simplify the method, reduce the complexities, and improve the accuracy based on the differential diagnostics of retinoblastoma at the nonneoplastic pathologies of eyes of primary patients, the migration capacity of peripheral blood leukocytes is determined, as an immunology reaction, in the presence of water soluble polypeptides extracted from the tumor retinoblastoma tissues (50 - 100 kD in molecular mass and at 100 to 200 μg per ml protein concentrations per 2.5 x 10^5 leukocytes); the migration indices (MI) are calculated 16 hrs after the beginning of the reaction; retinoblastoma is diagnosed at MI lower than 95 %; at MI values 95 % or higher the patients are diagnosed as having nontumor pathologies of eyes.